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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99203264.9

PRIORITY DOCUMENT

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A ONE STEP TEST FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

Field of the invention

The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs.

Background of the invention

The presence of antimicrobial residues in food and feed is a growing concern among the consumers due to health-related problems and the increase of drug resistant bacteria. Antibiotics are not only applied as medication, but certainly in case of poultry also widely used as antimicrobial growth promoting substances. It is well known that eggs may contain too high concentrations of antimicrobial residues. In most countries, such as the countries of the European Union, Canada and the United States, Maximum Residue Levels (MRL) are regulated by legislation.

Test methods to detect antimicrobial residues in body liquids such as milk or urine such as microbial inhibition tests (e.g. agar diffusion tests) or methods making use of selective binders (e.g. antibodies or tracers) have been known for a long time. Examples of microbiological test methods have been described in GB-A-1467439, EP 0005891, DE 3613794, CA 2056581, EP 0285792 and US 5494805. These descriptions all deal with ready to use tests that make use of a test organism. The test organism is mostly imbedded in an agar medium, which may contain an indicator, a buffer solution, nutrients and substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way.

Examples of suitable test organisms are strains of *Bacillus, Streptococcus* or *E.coli.* In general the principle of these tests is that when antibacterial compounds are present in a sample in a concentration sufficient to inhibit the growth of the test organism the color of an acid/base or redox indicator will stay the same, while when no inhibition occurs the growth of the test organism is accompanied by the formation of acid or reduced metabolites that will change the color of the indicator.

These test methods are suitable for the detection of antimicrobial residues in body liquids. However up to now detection of antimicrobial residues in eggs was not possible due to the presence of antimicrobial substances, such as lysozyme, which are naturally present in rather high concentrations in eggs. These inhibiting substances interfere with the micro-organism of the test leading to false positive results.

In case of e.g. a milk or urine sample inhibiting substances such as lysozyme or lactoferrin can be inactivated rather easily by heating the sample, e.g. at 80°C for 10 minutes (Vermunt et. al., Netherlands Milk and Dairy Journal 47: (1) 31 – 40 (1993)), or by using well known dialysis methods (van Wall, Archiv für Lebensmittelhygiene 29: (6) 235 (1978)). After this pre-treatment the liquid sample can be used for further testing simply by following the procedures of the test. In case of a Delvotest® type of test (EP 0005891) the liquid sample can be added directly to the test, after which the test is incubated.

However heating of an egg sample at temperatures sufficient to inactivate inhibiting substances of the egg, such as lysozyme, always leads to coagulation of the sample. It was believed that such samples are not suitable for further processing anymore.

Up to now after heating at a temperature sufficient to inactivate antimicrobial substances other than antimicrobial residues, the antimicrobial residues to be detected have to be extracted from the coagulated egg sample. These extraction methods not only cost a lot of time and extra handling but even worse always lead to loss of at least

part of the antimicrobial residues, if present in the sample (Inglis et. al., Journal for the Association of Official Analytical Chemists 61: (5) 1098 – 1102 (1978); Katz et. al., Journal of the Association of Official Analytical Chemists 61: (5) 1103 – 1106 (1978); Janetschke et. al., Monatshefe für Veterinaermedizin 34: (21) 824 – 826 (1979); Steiner, Monatshefe für Veterinarmedizin 45: (11) 382 – 386 (1990)). This may lead to false negative results and therefore antibiotics in consumer eggs, which of course is unacceptable from a health point of view. Moreover laboratories executing studies to the presence or absence of antimicrobial residues in foods are limited by the time available to execute these studies. With the present time consuming methods only a very limited amount of egg samples can be examined. Further these assays can only be executed in well-equipped laboratories and by well-educated persons, which is also a limited factor.

It can be concluded that up to now no suitable test method for the detection of antimicrobial residues in egg samples is available. The present methods are unreliable, time consuming and may lead to both false positive and false negative results, which leads to unacceptable amounts of antibiotics in the food chain and to economic losses.

Detailed description of the invention

The present invention discloses a reliable and very simple to carry out, one-step test for the detection of antimicrobial residues in eggs.

Unexpectedly it has been found that when an egg sample is added to the test after which the test is incubated for a sufficient time at a sufficient temperature to inactivate the natural inhibiting compounds of the egg, the test can be incubated directly after heating to determine the presence or absence of antimicrobial residues.

It has been surprisingly found that antimicrobial residues diffuse directly from the coagulated egg sample into the test system and that

therefore additional extraction methods to obtain the antimicrobial residues from the coagulated egg sample are not required anymore.

Surprisingly we also found that heating of the test containing the sample before incubation reduces the test duration considerably. This might be caused by activation of the spores present in the agar matrix.

In one aspect, the present invention provides a one step method for detecting antimicrobial residues in eggs and inactivation of the natural inhibiting substances of the egg sample. The test can be executed using the following methods:

(1) A sample of the egg is obtained by making a hole in the egg of e.g. approximately 1-2 square cm, prick the egg-yolk, place the egg with the hole down on a bottle, after the egg is empty the bottle is closed and the sample is homogenized by shaking. Alternatively of course any other method known in the art to obtain a sample of the total egg, egg white or egg yolk can be used;

(2) Add a sufficient amount of the egg sample to be tested to the test using well known methods;

(3) Heat the test, e.g. for approximately 10 minutes at 80° C, to inactivate the natural inhibiting substances (e.g. lysozyme), to activate the spores and to coagulate the egg sample;

(4) Incubate the test following the standard procedures of the test and read the result.

In case of conventional microbial inhibition tests, e.g. Delvotest® (test obtainable from DSM N.V. Holland) type of tests, inactivation of the natural inhibiting compounds present in the egg sample, e.g. lysozyme, and activation of the spores of the test organism is preferably achieved by heating for 5 - 15 minutes at 75° C – 85°C. Alternatively any other temperature / time treatment, which is sufficient to obtain said effects, can be used. The exact requirements depend e.g. on the condition of the

sample (e.g. the starting temperature, the volume of the sample, whole egg, egg white or egg yolk); the type of test (e.g. microbial inhibition tests or assays based on selective binders (e.g. antibodies or tracers)); the microorganism used in the test (e.g. thermophilic or non-thermophilic *Bacillus* or *Streptomyces* species). Of course it should be take care of that the heat treatment will not inactivate the antimicrobial residues to be detected. The heat treatment can be executed using any method known in the art, e.g. by heating in a water bath.

In a further aspect, the invention provides test units for carrying out the method of the invention. These test units contain the test and are suitable to execute the method of the invention: add the egg sample, heat to inactivate the natural inhibiting compounds of the sample and optionally activate the spores, incubate the test and read the results.

Examples of units useful for the purpose of the invention are transparent tubes, single or in a set or combined to a block of translucent material provided with a number of holes shaped therein. The test unit may contain solidified agar medium having therein said agar, optionally buffered; a test organism (e.g. a strain of *Bacillus* or *Streptococcus*) at sufficient colony forming units; nutrients for growth of said organism; an indicator (e.g. an acid-base or redox indicator); optionally substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way. All ingredients may optionally be added to the test as a separate source, for example as a tablet or paper disc.

The test units preferably have determined sizes. This is because of the reliability of the test. In case of a test based on agar diffusion technology preferably tubes are used. The test unit will preferably be high enough to contain an amount of agar medium and sample corresponding to preferably a height of 3-30 mm, more preferably 5-15 mm. The internal cross-sectional dimension of the test units is preferably 1-30 mm, more preferably 5-15 mm. The test units are preferably closed air tight during storage in which conditions they may be stored for at least several

months. Of course any other test unit suitable for executing the method of the invention is included in this invention.

The volume of the agar medium in the test unit is determined by the height of the test unit, the internal cross-sectional dimension of the test unit and the percentage of the volume of the test unit, which is filled with the agar medium. The volume of the agar medium is preferably 10 µl - 5 ml, more preferably 100 µl – 1 ml.

Any test suitable for the method of the invention is included in this invention. Examples are described in GB-A-1467439, EP-0005891, DE-3613794, CA-2056581, EP-028579 and US 5,494,805 which are incorporated herein by reference. Examples are tests in which selected sensitive microorganisms are used, e.g. microbial agar diffusion tests, and tests based on selective binding of the compound to be detected. Selective binding can be achieved using the well-known antibody technology or by using specific tracers. An example of a specific tracer is the penicillin binding protein, which is used in e.g. the Delvo-X-Press® for detecting beta-lactams.

Examples of suitable microbial agar diffusion tests are tests in which species of *Bacillus, Streptococcus or E. coli* are used. Preferably thermophilic species, e.g. *Bacillus stearothermophilus* and *Streptococcus thermophilus* are used. Examples of preferred strains are *Bacillus stearothermophilus var. calidolactis C953* (deposited with the Laboratory of Microbiology of the Technical University of Delft under the accession number LMD 74.1 in 1974 and with the Centraal Bureau voor Schimmelcultures (CBS), Baarn under the accession number CBS 760.83 in 1983 were the strain is available to the public) and *Streptococcus thermophilus T101* (DSM 4022, deposited on March 3, 1987). Both strains are very sensitive to antimicrobial compounds, especially chemotherapeutics such as sulfa compounds and antibiotics such as penicillins and tetracyclines. *E.coli* strains or other suitable gram-negative bacteria can be used for the detection of e.g. quinolones.

Bacillus stearothermophilus var. calidolactis C953 and *Streptococcus thermophilus T101* are fast growing and have the advantage that they are thermophilic. For example the optimum growth temperature of said *Bacillus* strain is between 50° and 70°C. The test organism is therefore very suitable for a test according to the invention as it is not killed of by heating to inactivate the natural inhibiting compounds of the egg sample.

When the test organism is a *Bacillus* strain, it is preferably incorporated into the agar medium in the form of a spore suspension which may be prepared and incorporated into the agar medium prior to solidification by known methods (see for example Gb-A-1467439). When the test organism is a *Streptococcus* strain, the bacteria are preferably incorporated into the agar medium in the form of bacterial cells which may be prepared according to known methods (see for example EP 0285792). The concentration of the test organism in the agar medium is preferably between 10^5 and 10^{10} colony forming units per ml of agar medium.

Suitable nutrients to enable multiplication of the test organism in the absence of antimicrobial residues are for example assimilable carbon sources (e.g. lactose, glucose or dextrose), assimilable nitrogen sources (e.g. peptone) and sources of growth factors, vitamins and minerals (e.g. yeast extract).

The growth of the test microorganism can be detected using well known methods, preferably by color change of the agar medium of the test sample. Mostly a color indicator, preferably an acid-base or a redox indicator, is used. Examples of suitable acid-base indicators are bromocresolpurple and phenolred. Examples of suitable redox indicators are brilliant black, methylene blue, toludine blue and nile blue. Also combinations of two or more indicators can be used.

Optionally the sensitivity of the test may be altered by adding certain substances, by changing the test conditions such as pH or

concentration of buffering substances or agar or by varying the ration of the volumes of agar and egg sample. Examples of substances that may be added to the test system to change sensitivity are nucleosides such as adenosine and antifolates such as trimethoprim, ormethoprim and tetroxoprim, which improve the sensitivity of the test organism to sulfa compounds. Salts of oxalic acid or hydrofluoric acid may be added to improve the sensitivity to tetracyclines. Cysteine may be added to diminish the sensitivity to penicillins. In this way the sensitivity of the test organism is adjustable to the antimicrobial compounds to be tested.

The amount of egg sample (whole egg, egg white or egg yolk of any species, preferably poultry) to be added to the test depends on the test system. For microbial diffusion tests 0.01 – 1.0 ml, preferably 0.05 – 0.5 ml is added to the test using well-known methods. After addition of the egg sample the test is heated to inactivate the natural antimicrobial compounds, e.g. lysozyme, of the egg sample. Preferably the test is heated for 2 – 20 minutes at 70°C – 100°C, more preferably the test is heated for 10 - 15 minutes at 75°C – 85°C or for 2 – 6 minutes at 100°C. It will be appreciated that any other time / temperature treatment, which is sufficient to inactivate the natural inhibiting compounds of the egg without inactivating the antimicrobial residues to be detected, may be used.

After the heat treatment the test is incubated following the instructions of the producer. The incubation time of the test is dependent on the circumstances. In case of an agar diffusion tests using *Bacillus stearothermophilus* the test is incubated in a water bath or block heater at 60°C – 70°C, preferably at 62°C – to 65°C. Results may be obtained after 1.5 to 4 hours, preferably from 2.5 to 3.5 hours. In case of tests using selective binders, such as antibodies or tracers, the results may be obtained within 30 minutes.

The method described in this invention is very simple to carry out, so that persons who perform the test do not have to be specially educated.

All documents mentioned in this application are herein incorporated by reference to the same extent as if each individual application or patent was specifically and individually indicated to be incorporated by reference.

Example 1

Preparation of a whole egg sample

To obtain an egg sample for examination on the presence or absence of antimicrobial residues a hole of approximately 1-2 cm^2 is made in the egg, the egg yolk is pricked and the egg is placed with the hole down on a bottle which allows the egg white and egg yolk to drip into the bottle. After the egg is empty, the bottle is closed and the sample is homogenized by shaking. Now the sample is ready for further studies.

Example 2

Inactivation of natural inhibiting compounds of the egg and examining the samples on Delvotest®

Samples of 5 eggs (duplo), which did not contain antimicrobial residues, were obtained according to the method described in Example I. To inactivate the natural inhibiting compounds present in the egg sample, 100 μl of each of the 5 samples was added on Delvotest® ampoules. The test was produced according to the methods described in EP 0005891 with the nutrients present in the agar. After heating for 10 minutes at 80°C in a waterbath, the ampoules were immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. After 140 minutes the colour of all tests turned from purple to yellow, indicating that no antimicrobial rediues were present.

The control samples were not heated at 80°C for 10 minutes, but directly placed on the ampoule. These tests remained purple for at least 4 hours.

These results clearly demonstrate that natural inhibiting compounds of the egg sample inhibit the test leading to false-positive results. When the sample is heated as described above, the activity of the natural inhibiting compounds is eliminated and no false-positive results are observed anymore.

Example 3

Determination of the sensitivity of the Delvotest® according to the method described in this invention using spiked samples

Egg samples were obtained according to the method described in Example I. Said samples were spiked by adding Penicillin G (0 and 4 ppb) or Sulphadiazine (0 and 100 ppb) using well known methods. Said egg samples were added to Delvotest® ampoules (see Example II) according to the method described in this invention: heat for 10 minutes at 80°C, after which the ampoules are immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. The results were read as soon as the blanco turned to yellow (after 140 minutes). The blanco samples were negative, while the samples spiked with 4 ppb Penicillin G and 100 ppb sulphadiazine remained purple (positive).

These results clearly demonstrate that the method descibed in this invention is suitable for detecting antimicrobial residues in egg samples.

CLAIMS

1. A process to detect antimicrobial residues in an egg sample comprising
   - taking a sufficient amount of sample of the egg to be tested,
   - adding the sample on a test suitable to detect antimicrobial residues,
   - heating the sample to inactivate lysozyme present in the egg sample and to coagulate the egg sample,
   - incubating the test, and
   - determining the presence of the antimicrobial residue.

2. A process according to claim 1 wherein the test comprises a test organism, nutrients and one or more indicators present in an agar medium.

3. A process according to claim 2 whereby the degree of growth or inhibition of growth of the test organism is determined indicating the absence or presence of the antimicrobial residue.

4. A test unit which comprises
   - a test for the detection of antimicrobial residues and
   - a coagulated egg sample added to the test.

A ONE STEP TEST FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

ABSTRACT

The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs. A one step test method is described in which residues of antimicrobial compounds such as antibiotics are detected while inhibiting compounds naturally present in samples obtained from eggs, which may interfere with the test, are inactivated.